amplified and were verified to be homogenous through DNA sequencing. Cells were prepared for binding as was described for biopanning procedures. Preparations of plaque-purified and titered phage ($1x10^{11}$) were incubated in serum-free Opti-MEM on either CEF or CEF+hTfR cells at 4°C for 1 hour. The cells were washed repeatedly with Opti-MEM and bound phage were eluted with low pH buffer and subsequently titered. In competition studies, holo-transferrin (Calbiochem, La Jolla, CA) or synthesized peptides were added to CEF+hTfR cells prior to addition of the phage for 1 hour at 4°C. Multiple trials were completed and average titers and standard deviations determined. The titers determined on CEF+hTfR cells were divided by the titers determined on CEF cells and multiplied by 100 to yield fold over control data points.

Modified GFP Constructs: Transferrin from human serum, bovine serum albumin (BSA), and purified wild-type GFP (wtGFP) were obtained from Sigma (St. Louis, MO) and Clontech (Palo Alto, CA), respectively. The tagged GFP genes were generated by the PCR with template DNA Clontech's GFP vector. The PCR reactions were carried out in a Perkin Elmer Cetus DNA Thermal Cycler for 30 cycles of 95°C, 1 minute; 55°C, 1 minute; and 72°C, 1 minute.

HAIYPRH (Seq. ID No. 1)-tagged GFP PCR oligonucleotides were:

Upstream: 5'-TCTAGATCTGATGAGTAAAGGAGAAGAA-3' (Seq. No. 7)

Downstream: 5'-TTAAAGCTTTTAATGGCGCGGATAGATCGCATGTTTGT AGAGCTCATCCATGCC-3' (Seq. No. 8)

THRPPMWSPVWP (Seq. ID No. 2)-tagged GFP PCR oligonucleotides were:

Upstream: 5'-TCTAGATCTGATGAGTAAAGGAGAAGAA-3' (Seq. No. 7)

Downstream: 5'-TAAAGCTTTTACGGCCACACCGGGCTCCACATCGGCGGG CGGTGGGTTTTGTAGAGCTCATCCATGCC-3' (Seq. No. 9)

The PCR products were purified with the Qiagen Gel Extraction Kit (Qiagen, Valencia, CA), and cut with BglII and HindIII restriction enzymes (Roche, Nutley, NJ), and subcloned into the pET-32a(+) bacterial expression vector (Novagen, Madison, WI). The resulting expression vector was verified using a modified Sanger sequencing method. The tagged GFP expression plasmids were transformed into BL21/DE3 E. coli and expression was induced for 3-4 hours with 1 mM isopropyl-ß-D-thioglactopyranoside (IPTG) when the culture $O.D._{600}$ = 0.5. Cells were pelleted, then resuspended in phosphate buffer with 20 mM imidazole followed by passage through a French press at 10,000-15,000 psi. Cell lysates were passed over a PisTrap nickel column (Amersham Pharmacia Biotech, Piscataway, NY). The column was washed and finally eluted using an imidazole gradient. The purified protein was assayed by SDS-PAGE followed by Coomassie staining and Western blot analysis with a GFP monoclonal antibody (Clontech). ECL development was carried out as per the manufacturer's instructions (Amersham Pharmacia Biotech). Protein concentrations were determined with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA).

Immunofluorescence:: CEF+hTfR cells were grown on glass coverslips to 50% to 75% confluence. The coverslips were washed and incubated in serum-free Opti-MEM media at 37°C for 1 hour. Then 2 µg of wild-type GFP (Clontech), HAIYPRH (Seq. ID No. 1)-tagged GFP, THRPPMWSPVWP (Seq. ID No. 2)-tagged GFP, or Texas-Red

Tf (Molecular Probes) was applied to cells in serum-free Opti-MEM media for 1 hour at 4°C or 37°C. Cells were washed with Opti-MEM, then fixed in 3% formaldehyde for 30 minutes at 4°C. Alternatively, the cells were acid-washed with 0.2 M glycine-HCl, Ph 2.2, prior to fixation. A GFP monoclonal antibody (Clontech) was used in conjunction with an Oregon-Green Goat Anti-Mouse (Molecular Probes) to augment GFP fluorescence. All slides were counterstained with DAPI (2(4Amidinophenyl)- 6indole carbamidinedihydrochloride) (Sigma). The microscopic slides were mounted in Prolong™ antifade medium (Molecular Probes). Images were captured on an AX70 microscope with Olympus Camera (Olympus, Melville, NY) and analyzed with ESPRIT software (Life Science Resources, Cambridge, England). Final figures were assembled using Microsoft Power Point (Microsoft Corp., Redmond, WA). For colocalization studies, CEF+hTfR were incubated with 2 μg/ml GFP fusion protein and 2 μg/ml of Texas-Red Tf for 1 hour and processed as described above.

Standard Analysis: Purified proteins (transferrin, wtGFP, GFP-HAIYPRH (Seq. ID No. 1) and GFP-THRPPMWSPVWP (Seq. ID No. 2) were labelled with ^{125}I to a specific activity of 1-2 μCi/μg with CPM/μg determined by a gamma counter and Bradford assay. CEF+hTfR cells were plated in duplicate at a density of $7.5x10^4$ cells/well in 24 well dishes and grown overnight. Cells were washed and incubated in serum-free Opti-MEM for 1 hour at 37°C. Cells were placed on ice with the various amounts of labelled protein in a total of 200 μl of cold 0.1% BSP in phosphate buffered saline

(PBS). After 1 hour, the unbound protein was removed and cells were washed 4 times with 0.1% BSA in PBS. 1 M NaOH was added to lyse the cells for determining the bound fraction. Both unbound and bound fractions were counted in a gamma counter and binding affinities were determined using Scatchard analysis. Studies were repeated 3 times and yielded comparable binding affinities for all proteins tested.

Example 1:

The biopanning procedure with chicken embryo fibroblast cells was performed as described above. The procedure used both negative and positive binding steps to isolate specific peptide sequences that bind the hTfR.

After the cells were incubated in serum-free media Opti-MEM at 37°C for 1 hour to remove Tf found in the serum, ice-cold serum-free Opti-MEM media was applied and the cells were held at 4°C throughout the selected process to prevent internalization of the receptor. The original phage library containing $2x10^{11}$ phage was applied to CEF cells for 2 hours. Unbound phage were transferred to another well of CEF cells for an additional hour, before transferring the unbound phage to a well of CEF+hTfR cells. After extensive washing, the bound phage were removed with low pH buffer and subsequently neutralized. The eluted phage were titered and amplified in E. coli. After each amplification step multiple plaques were selected for sequencing. The amplified eluted phage were applied to CEF cells to begin the biopanning process again. This cycle was carried out 10 rounds for the 7-mer peptide library

and 7 rounds for the 12-mer peptide library to achieve significant enrichment of a single sequence above all others. Sequencing of individual phage plaques allowed for the monitoring of sequence convergence during multiple rounds of biopanning. Phage titers of total phage eluted were determined and were noticed to increase after each round of biopanning. The most prominent sequence selected from the 7-mer library was HAIYPRH (Seq. ID No. 1) (7-mer) while the 12-mer library converged to the sequence of THRPPMWSPVWP (Seq. ID No. 2) (12-mer). There were no other sequences that arose consistently throughout the biopanning procedure.

Phage that did not bind the CEF cells were applied to CEF+hTfR cells and the bound phage were eluted with low pH buffer. The eluted phage were amplified for additional rounds of biopanning. Between each round, the phage were titered and sequenced to monitor convergence of sequence.

Example 2:

As an initial test to determine whether the isolated phage bound to hTfR, a phage binding study was performed. Homogeneous pools of five different isolates from the 7-mer phage and five isolates from the 12-mer phage were each amplified, purified and verified by DNA sequencing. Individually, 10^9 phage were applied to CEF or CEF+hTfR cells. Phage were bound to CEF or CEF+hTfR cells for 1 hour at 4°C, then washed extensively with Opti-MEM to remove unbound phage. Bound phage were eluted with low pH buffer, neutralized, and titered on a lawn of E. coli. Titering each phage on both cell types was repeated three times, and average titers and

standard deviations were determined. Considering the 7-mer sequences, it was found that significantly higher titers were obtained only with HAIYPRH (Seq. ID No. 1) phage bound to CEF+hTfR cells when compared to other phage tested. In studies relating to the 12-mer sequences, it was found that the THRPPMWSPVWP (Seq. ID No. 2) phage had higher titers on CEF+hTfR cells than the other 12 amino acid phage tested. On CEF cells, which do not express hTfR, all phage tested bound at the same low efficiency. A low level of non-hTfR dependent binding is expected, due to interactions between phage coat proteins and the various proteins on the surface of chicken embryo fibroblast cells. Titering studies demonstrated that phage containing either peptide sequence HAIYPRH (Seq. ID No. 1) or THRPPMWSPVWP (Seq. ID No. 2) bound CEF+hTfR cells more efficiently than any other phage tested and that this higher binding depends on the presence of human transferrin receptor.

Example 3:

Competition studies were conducted to determine whether the two phages bound the same region of the hTfR as serum Tf itself. It was found that the titers of HAIYPRH (Seq. ID No. 1) or THRPPMWSPVWP (Seq. ID No. 2) phage bound to CEF+hTfR cells in the presence of various added peptides or Tf were significant. The HAIYPRH (Seq. ID No. 1) phage was competed away to background levels only by the HAIYPRH (Seq. ID No. 1) peptide and not by the scrambled sequence of IRHPHYA (Seq. ID No. 3). The 12-mer THRPPMWSPVWP (Seq. ID No. 2) phage was only competed by the THRPPMWSPVWP (Seq. ID No. 2) peptide and not by the scrambled 12-

mer sequence PWRPSHPVWMPT (Seq. ID No. 4). Other peptides tested failed to compete away the phage from binding the cells. Interestingly, the binding of either phage was unaffected by the presence of Tf, suggesting that each phage sequence has a different binding site on the hTfR. Due to a synthesis error, a peptide with the sequence HAIYPNH (Seq. ID No.14) was also synthesized. Competition studies were completed with this peptide which disclosed no effect on the HAIYPRH (Seq. ID No. 1) phage binding. The result suggests that the 7-mer phage binding depended on the arginine in the original HAIYPRH (Seq. ID No. 1).

Example 4:

To evaluate the sufficiency of capacity of the peptides to mediate uptake of carrier protein, GFP fusion proteins were prepared. Immunofluorescence was used to determine if the GFP-peptides fusion constructs were internalized using the following assay. GFP-peptide constructs were cloned with a C-terminal peptide addition of either HAIYPRH (Seq. ID No. 1) (GFP-HAIYPRH) or THRPPMWSPVWP (Seq. ID No. 2) (GFP-THRPPMWSPVWP). These constructs were expressed and purified to greater than 95% by Coomassie staining. Purified proteins were applied to CEF+hTfR cells at 4°C (which prevents endocytosis) or at 37°C. Cells were washed with Opti-MEM, fixed and processed as described under the Materials and Methods section above. Alternatively, the cells were washed with low pH buffer prior to fixation. This acid wash determined whether the protein was endocytosed by removing proteins bound at the cell surface.

Immunofluorescence microscopy was used to follow binding and internalization of the wtGFP, GFP fusion proteins and transferrin to CEF+hTfR cells. Wild-type GFP was used as a negative control, while Tf conjugated to the Texas-Red fluorochrome was used as a positive control. The conjugation of Texas-Red to Tf has been shown previously not to diminish interaction with the hTfR. In all studies, cell nuclei were counterstained with DAPI.

Immunofluorescence images of the localization of various proteins applied to CEF+hTfR cells at either 4°C or 37° were studied. At 4°C, endocytosis was blocked so that all proteins remain at the cell surface, and an acid wash removes all cell surface bound proteins. When immunofluorescence of the various proteins was studied on CEF+hTfR cells which had been incubated at 37°C for one hour, localization of GFP-HAIYPRH (Seq. ID No. 1), GFP-THRPPMWSPVWP (Seq. ID No. 2) or Texas-Red Tf was found on cells that had not been exposed to acid wash. The total fluorescence shown could result from both cell surface and endocytosed proteins. There was minimal binding of wtGFP even without an acid wash.

The cells that had undergone a low pH buffer wash to enable identification of proteins that had been endocytosed were evaluated. While wtGFP was unable to be endocytosed into CEF+hTfR cells, both GFP-HAIYPRH (Seq. ID No. 1) and GFP-THRPPMWSPVWP (Seq. ID No. 2) showed a speckled pattern of fluorescence typical of endocytosed ligands. The Texas-Red Tf was readily endocytosed into the CEF+hTfR cells and produced a spotted pattern similar to that seen with the two GFP fusion proteins.

In separate studies at 4°C or 37°C, CEF cells were used for immunofluorescence binding assays and neither of the GFP fusion proteins or transferrin bound or internalized these cells, as was expected, since these CEF cells lack the hTfR. Immunofluorescent internalization studies were also performed with Hela cells and yielded identical result to CEF+hTfR cells.

Example 5:

The phage titering experiments demonstrated that neither peptide sequence competed with Tf or hTfR binding. Co-localization studies were conducted with both GFP-Peptide and Texas-Red Tf constructs. Cells were incubated at 37°C for 1 hour with Texas-Red Tf and either GFP-HAIYPRH (Seq. ID No. 1) or GFP-THRPPMWSPVWP (Seq. ID No. 2). Cells were acid washed immediately, fixed and stained with DAPI. Images were captured using the appropriate filter and overlaid with images captured with the DAPI filter. Merging GFP, Texas-Red Tf and DAPI images yielded the co-localization images. The fluorescent patterns of the GFP fusion proteins and Tf were identical after acid wash. This result indicated that the GFP-peptides were internalized and bound in the same intracellular compartment as Tf.

Example 6:

Purified transferrin, wtGFP, GFP-HAIYPRH (Seq. ID No. 1) and GFP-THRPPMWSPVWP (Seq. ID No. 2) were labelled with ^{125}I on tyrosine residues to a specific activity of 1-2 $\mu Ci/\mu g$. Serial dilutions of labelled proteins were incubated with CEF+hTfR cells on ice in PBS-0.1%BSA in duplicate wells. After 1 hour, the unbound fraction was

removed and cells were washed four times. Cells were removed from the well with 1N NaOH. The unbound and bound fractions were counted in a gamma counter and fmoles of bound and unbound were calculated. Scatchard plots were derived by plotting bound versus bound/free of an average value generated by the duplicate wells. A best of fit line was generated using the Excel program (Microsoft Corp.) and the binding affinities were determined by the slope of the plotted lines. Repetitive trials produced comparable binding affinities. The affinity of Tf was found to be 2.7×10^{-9}, similar to previous reports. The affinity for wtGFP and GFP-HAIYPRH (Seq. ID No. 1) were determined to be nominal at 2.4×10^{-4}M and 3.6×10^{-4}M, respectively. This low affinity of GFP-HAIYPRH (Seq. ID No. 1) was attributed to the ^{125}I labelling of the tyrosine residue in the peptide, which could block this peptide's interaction with the hTfR. However, GFP-THRPPMWSPVWP (Seq. ID No. 2) was shown to have 2.3×10^{-8}M affinity for CEF+hTfR cells, indicating that its affinity was only 10-fold lower than the native Tf ligand.

Peptides containing the sequences HAIYPRH (Seq. ID No. 1) and THRPPMWSPVWP (Seq. ID No. 2) can be used to target viral vectors, as well as proteins, to the endocytic pathway via the hTfR. Competition studies suggest that transferrin, the 7-mer sequence and the 12-mer sequence all bind unique sites on the hTfR, since they each failed to significantly compete with each other for hTfR binding. This finding suggests further advantages for use of these peptides for transduction of therapeutic ligands, since there is no disruption of transferrin's delivery of iron to cells.

Due to the characteristics and expression pattern of the hTfR, ligands specific for this receptor may be used as targeting agents with antigen as well as diagnostic agents such as imaging agents or radioisotopes. It has been shown that early endosomes are essential for the proper endocytosis, sorting and presentation of antigen by major histocompatibility class II. The targeting of antigens to the hTfR enhances antigen entry into the endocytic pathway and boosts antigen presentation.

It is possible to conjugate the peptides of the invention to liposomes or viral vectors containing active agents such as chemotherapeutics. (See Eavarone, et al, "Targeted Drug Delivery to C6 Glioma by Transferrin-coupled Liposomes", Proceedings of the World Biomaterials Congress 2000, (John Wiley and Sons, Inc.) (2000)). Alternatively, chemotherapeutics may be conjugated directly with the peptides of the invention for targeting agents to transferrin receptor-rich cells. Because the peptides of the invention do not interfere with binding of human transferrin to the hTfR, different agents may be administered wherein one conjugate targets the hTfR uses transferrin as the targeting agent and another conjugate targets the hTfR using a peptide of the invention as a targeting agent.

Example 7:

Transferrin receptor binding peptide sequences to adenovirus proteins in accord with the teachings of U.S. Patent 6,312,699, which is incorporated herein by reference in its entirety. As described in example 2 of US Patent 6,312,699, short peptide

ligands such as HAIYPRH (Seq. ID No. 1) and THRPPMWSPVWP (Seq. ID No. 2) are fused onto the carboxyl-terminus of the adenovirus fiber protein. Oligonucleotides encoding these amino acid sequences are designed and synthesized and annealed together for cloning into the unique BamHI restriction endonuclease cleavage site in plasmid pTKgpt-3S (cited in example 2 of US Patent 6,312,699). Examples of such oligonucleotides are:

For HAIYPRH (Seq. ID No. 1):

Sense: 5′ GA TCC CAT GCG ATC TAT CCG CGC CAT TAA 3′(Seq. ID No. 10)

Antisense: 5′ G ATC TTA ATG GCG CGG ATA GAT CGC ATG G 3′ (Seq. ID No. 11)

For THRPPMWSPVWP (Seq. ID No. 2):

Sense: 5′ GA TCC ACC CAC CGC CCG CCG ATG TGG AGC CCG GTG TGG CCG TAA 3′ (Seq. ID No. 12)

Antisense: 5′ G ATC TTA CGG CCA CAC CGG GCT CCA CAT CGG CGG GCG GTG GGT G 3′ (Seq. ID No. 13)

These oligonucleotides are designed with BamHI cohesive ends that can be cloned into the BamHI cleavage site developed in Example 2 of US Patent 6,312,699. The specific amino acid sequence added to fiber in Example 2 was designed to extend the new transferrin receptor-binding ligand away from the bulk of the fiber protein, increasing its accessibility to the new receptor molecule. The fiber protein, modified to include a linker and a ligand, could still form a trimer.

The non-viral ligands can be attached to the carboxyl terminus of the fiber protein via a peptide linker by expression of

a genetically engineered nucleic acid sequence encoding the fiber protein, linker, and ligand. Alternatively, one could use PCR mutagenesis to introduce these two sequences into plasmid pTKgpt-3S, using synthetic oligonucleotides as in example 4 of the cited patent.

Example 8:

The Tf receptor binding peptides can be used to enhance antigen delivery in antigen-presenting cells. These peptide sequences are applied to increase the potency of vaccines, since antigen-presenting cells often take up the antigens contained in vaccines poorly. To enhance antigen delivery and, therefore, antibody and cytotoxic T cell responses, these peptides are chemically coupled to the antigen of interest or prepared as a recombinant protein that contains these Tf receptor-binding peptides. For preparation of the recombinant antigen containing the Tf receptor binding peptide, coupling is accomplished using standard recombinant DNA techniques as in other examples provided (for example, fusions of HAIYPRH (Seq. ID No. 1) and THRPPMWSPVWP (Seq. ID No. 2) to GFP or adenovirus fiber proteins.) The recombinant proteins can be expressed in any number of protein expression systems including bacterial, baculoviral, and mammalian expression systems.

For chemical conjugation of the Tf receptor binding peptides, the peptides are coupled using chemical crosslinkers such as succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC; Piece Chem. Co., Rockford, IL). Obviously, any chemical crosslinker

could be used for this purpose. In our applications, we have coupled 10 mg of antigen to a 5 to 30-fold molar excess of SMCC in 50 mM Hepes buffer (pH 7.4) for 1 h at room temperature.

Example 9:

SMCC-modified antigen is purified by gel filtration to remove the unbound crosslinker. Using this particular crosslinker, peptides are prepared with an amino-terminal linker sequence with a cysteine residue followed by a nonspecific linker sequence (glycine-proline-glycine) to facilitate the coupling reaction. (The leader sequence can change depending on the nature of the crosslinker.) After the leader sequence, the 7- or 12-residue Tf receptor binding peptide is attached. The peptides are added to SMCC-modified antigens at same molar ratio as is used with the cross-linker. The reactions are incubated overnight at room temperature.

Reaction products are separated by gel filtration and the number of cross-linkers and/or peptides coupled to the antigen is determined by MALDI-TOF mass spectrometry. These Tf receptor binding peptide-modified antigens can then be used as a vaccine using standard vaccination protocols.

The advantage of the peptide-coupled antigens is that substantially less antigen will be required for inducing antibody-based responses. Since a number of peptides can be coupled to each antigen molecule, antigenic responses should be dramatically enhanced.

Exampl 10:

The peptides of the invention may also be coupled with chemotherapeutic agents. Using 2 equivalents of either peptide HAIYPRH (Seq. ID No. 1) or THRPPMWSPVWP (Seq. ID No. 2) or a combination of the two, to one equivalent of methotrexate the peptides of the invention are coupled to methotrexate using the methods of examples 8 and 9. The resulting product is formulated in buffered saline and administered to the patient in sufficient amount to provide a concentration of .3 to 5 μM in the serum when administred intravenously.

Example 11:

The methotrexate bound to the peptides of the invention is prepared as in example 10. However, the methotrexate bound to the peptides is then formulated in liposomal form for intravenous administration. Liposomal compositions may also be administred by mouth or directly to the affected tissue.

Examples of other antineoplastic agents that might be conjugated to the peptides of the invention, either directly or through conjugation to or incorporation in liposomes containing the sequences of the invention, such liposomes containing antineoplastic agents which may be bound to the peptides of the invention, to target cells rich in human transferrin receptors include (but are not limited to) cisplatin, nitrogen mustards (including chlorambucil), ethylenimines, methylmelamines, nitrosoureas (including carmustin, lomustine, etc.) and doxorubicin. The antineoplastic agents would be administered in accord with the methods usually used for the particular agent and

disease. However, because of the selective targetting of the agent by the peptides, lower dosage is required. (The lowering of dosage of the antineoplastic agent can be as much as 80%.) Furthermore, because the over-all dosage of the neoplastic agent can be decreased, the active agent can be administered for a longer period of time and more frequently than when the non-targetting agent is employed.

The compositions with the peptides bound to antigens or antineoplastic agents may be administered directly to the involved tissues. For example, in cases of maligancy of the respiratory tract, the agents may be administred by inhalation. In treating maligancies of the brain or spinal cord, the agents may be administered intrathecally. For oral administration, the peptide-bound agents may be administred in enteric coated dosage forms to prevent destruction in the stomach.